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<b>(21) International Application Number:</b> PCT/AU98/00882 <b>(22) International Filing Date:</b> 23 October 1998 (23.10.98) <b>(30) Priority Data:</b> PP 0120 30 October 1997 (30.10.97) AU <b>(71) Applicants (for all designated States except US):</b> COMMON-WEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION [AU/AU]; Limestone Avenue, Campbell, ACT 2601 (AU). MEAT & LIVESTOCK AUSTRALIA LIMITED [AU/AU]; Level 1, 165 Walker Street, North Sydney, NSW 2060 (AU). <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only):</b> BARENDSE, William, John [AU/AU]; 37 Ghost Gum Street, Bellbowrie, QLD 4070 (AU). <b>(74) Agent:</b> GRIFFITH, Hack; 509 St. Kilda Road, Melbourne, VIC 3004 (AU).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> ASSESSING LIPID METABOLISM <b>(57) Abstract</b> <p>This invention relates to methods and nucleic acid probes for assessing characteristics of lipid metabolism in animals, and in particular to methods of predicting fat levels in meat, milk, or other fat depots of animals. Thus the invention provides a method of assessing the fat metabolism characteristics of an animal, comprising the step of testing the animal for the presence or absence of one or more markers selected from the group consisting of: a) an allele of the 5' untranslated region of the gene encoding thyroglobulin; b) an allele of the DNA polymorphism CSSM34, associated with the gene encoding retinoic acid receptor gamma (RARG); c) an allele of the DNA polymorphism ETH10, associated with 11-cis, 9-cis retinol dehydrogenase (RDH5). The invention is particularly applicable to predicting deposition of fat in muscular tissue, which produces the characteristic "marbling" of meat, and to assessment of milk fat content. The methods of the invention are useful in selection of animals, particularly cattle, for ability to produce or high levels of marbling in meat, and to produce high or low levels of milk fat content.</p> <div style="text-align: right;"> <p><b>Tests of Association between DNA markers on chromosome 5 and marbling</b></p> <p>Gene Order          BM9025*          LALBA          CSSM34          BM900          ETH10          RM29          CSSM22          AGLA254          BM315          ETH2</p> <p>— Strong association at location of RARG            Location of RDH5</p> <p>* Yates correction for continuity due to many alleles with small expectations</p> </div>		

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**ASSESSING LIPID METABOLISM**

This invention relates to methods and nucleic acid probes for assessing characteristics of lipid metabolism in animals, and in particular to methods of predicting fat levels in meat, milk, or other fat depots of animals. The invention is particularly applicable to predicting deposition of fat in muscular tissue, which produces the characteristic "marbling" of meat, and to assessment of milk fat content. The methods of the invention are useful in selection of animals, particularly cattle, for ability to produce or high levels of marbling in meat, and to produce high or low levels of milk fat content.

**BACKGROUND OF THE INVENTION**

The manner in which animals metabolise fat is of considerable economic significance in agriculture and animal husbandry. In some markets the high content of fat in meat, in the form of small fat deposits or "marbling", is regarded as highly desirable, and to induce heavy marbling of meat in cattle in particular the animals are grain fed for at least a short period prior to marketing and slaughter. In other markets a very lean meat is preferred. Similarly, a high fat content of milk is usually regarded as desirable. This can be particularly important if the milk is to be used for cheese production, and so these factors are important not only in cattle but also in sheep and goats. Recently generation of transgenic animals which secrete valuable proteins into their milk has been achieved, and in order to reduce the costs of purification of the desired protein a low content of fat in the milk is desirable.

Thus there is a need for methods by which the propensity of animals, particularly bovids and other ungulates, to deposit fat in muscle or to secrete fat into milk can be assessed.

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Intramuscular or marbling fat is deposited in cattle between the fascicles of muscles, and usually develops when animals are fed a high calorie diet for a long time. The quantity of marbling fat is expressed  
5 either as a lipid concentration or as a standardised marbling score (eg. the Australian AUSMEAT standard). Unlike fat deposited in subcutaneous and renal depots, marbling fat is deposited continuously until relatively late in the development of the animal (Hood and Allen,  
10 1973; Ciazio *et al*, 1985), and the amount of this fat is strongly correlated with the number of fat cells or adipocytes found in the muscle fascicles. Although some of the factors that are important in the differentiation of adipocytes are known (Ailhaud *et al*, 1992; Smas and Sul,  
15 1995), the genetic factors that are involved in the difference between individuals in differentiation and development of the interfascicular adipocytes and deposition of fat were unknown, as were the genetic variants leading to a high or low marbling score.

20 To address this lack of information, we have obtained cattle samples from several breeds, the Angus, the Shorthorn and the Wagyu. These samples were readily differentiated due to their marbling score, with approximately half of the sample having a high marbling  
25 score and the other half of the sample having a low marbling score. We tested DNA markers from several regions of the bovine genome on the samples and the distribution of alleles was compared in the two groups.

Surprisingly, a significant association to  
30 marbling score was found with the anonymous DNA marker CSSM66. This marker had been assigned to bovine chromosome 14 (chr. 14) on the International Bovine Reference Family Panel (described in Barendse *et al*, 1997), with a location near the centromere. The gene for  
35 thyroglobulin (TG) is known to be located near this DNA marker (Barendse *et al*, 1997). TG is the molecular store for the thyroid hormones triiodothyronine and

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tetraiodothyronine, which have been implicated in the development of fat cells (Ailhaud et al, 1992; Darimont et al, 1993; Smas and Sul, 1995). TG has been sequenced in cattle (De Martynoff et al, 1987; Parma et al, 1987), and  
5 several DNA polymorphisms have been described previously (Georges et al, 1987). However, none of these polymorphisms is associated with fat or marbling.

We sought a polymorphism in the 5' untranslated region (5'UTR) of TG in cattle, since the transcriptional,  
10 and translational regulation of genes is mediated by the 5'UTR (Ptashne, 1988; Beato, 1989; Kozak, 1991).

A novel polymorphism in the 5'UTR of TG was identified, and shown to be correlated with marbling. This polymorphism can be used as a test to select animals for  
15 marbling performance, either as breeding stock or as animals to be fed for particular markets. Other characteristics of fat, such as fat thickness in other fat depots as well as fat percentage of tissues, including milk, are expected to be predicted by this marker, since  
20 the iodothyronines affect the general differentiation of adipocytes and since the influence of the level of the thyroid hormones on milk fat percentage is well known (Folley and Malpress, 1948). It is also expected that fat percentage of other mammalian species will be predicted by  
25 variation in the 5'UTR of the TG of those species.

In addition, we have surprisingly found significant associations between marbling score and the hitherto anonymous DNA markers CSSM34 and ETH10 on chromosome 5. CSSM34 is associated with retinoic acid  
30 receptor gamma (RARG), which is a known factor in the growth and differentiation of adipocytes. ETH10 is associated with retinol dehydrogenase 5 (RDH5), which catalyzes the interconversion of retinol and retinoic acid, and the level of retinol in the serum is directly related  
35 to intramuscular fat levels. The thyroid and steroid hormones such as thyroxine, retinol, and estrogen bind to a family of nuclear receptors with a similar set of hormone

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response elements. These nuclear receptors, such as RARG, initiate the transcription of genes, and are important elements in the growth, differentiation and specification of tissues. These elements are linked together  
5 structurally by similarities at the DNA sequence level.

#### SUMMARY OF THE INVENTION

In its general aspect the invention provides a method of assessing the fat metabolism characteristics of  
10 an animal, comprising the step of testing the animal for the presence or absence of one or more markers selected from the group consisting of:

- a) an allele of the 5' untranslated region of the gene encoding thyroglobulin;
- 15 b) an allele of the DNA polymorphism CSSM34, associated with the gene encoding retinoic acid receptor gamma (RARG); and
- c) an allele of the DNA polymorphism ETH10, associated with 11-cis, 9-cis retinol dehydrogenase (RDH5).

20 According to a first embodiment the invention provides a method of assessing the fat metabolism characteristics of an animal, comprising the step of testing the animal for the presence or absence of an allele of the 5' untranslated region of the gene encoding  
25 thyroglobulin.

Preferably the allele is allele 3, which indicates a high marbling score and/or high fat content of milk, or is allele 2, which indicates a low marbling score and/or low fat content in milk.

30 In a second embodiment the invention provides a method of identifying an animal with a high propensity for fat deposition in muscle (high marbling score), comprising the step of testing said animal for the presence or absence of allele 3 of the 5' untranslated region of the gene  
35 encoding thyroglobulin, and selecting those animals possessing the allele. Preferably the animal is also tested for the presence or absence of allele 2 of the

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5' untranslated region of the gene encoding thyroglobulin, and those animals possessing allele 3 and not possessing allele 2 are selected. Most preferably the animal is homozygous for allele 3.

5 In a third embodiment, the invention provides a method of identifying an animal with a low propensity for fat deposition in muscle, comprising the step of testing the animal for the presence or absence of allele 2 of the 5' untranslated region of the gene encoding thyroglobulin, and selecting those animals having allele 2. Preferably the animal is also tested for allele 3, and those animals having allele 2 but not allele 3 are selected. Most preferably the animal is homozygous for allele 2.

15 According to a fourth embodiment the invention provides a method of identifying an animal with a high propensity for fat deposition in muscle (high marbling score), comprising the step of testing the animal for the presence or absence of an allele of the DNA polymorphism CSSM34 associated with the gene encoding retinoic acid receptor gamma (RARG).

20 Preferably the allele is allele 2, which indicates a high marbling score. Preferably the animal is also tested for other alleles at the CSSM34 DNA polymorphism. For high marbling scores the animal is most preferably homozygous for allele 2. Allele 2 is 102 base pairs (bp) of DNA long.

30 According to a fifth embodiment the invention provides a method of identifying an animal with a low propensity for fat deposition in muscle, comprising the step of testing the animal for the presence or absence of an allele of the DNA polymorphism CSSM34 associated with the gene encoding retinoic acid receptor gamma.

35 Preferably the allele is allele 6, which indicates a low marbling score. Preferably the animal is also tested for other alleles at the CSSM34 DNA polymorphism. For low marbling scores the animal is most

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preferably homozygous for allele 6. Allele 6 is 112 bp of DNA long.

According to a sixth embodiment the invention provides a method of identifying an animal with intermediate propensity for fat deposition in muscle (low marbling score), comprising the step of testing the animal for the presence or absence of an allele of the DNA polymorphism CSSM34 associated with the gene retinoic acid receptor gamma.

10 Preferably the allele is one or more of alleles 1, 3, 4, and 5 which indicates an intermediate marbling score. Preferably the animal is also tested for other alleles at the CSSM34 DNA polymorphism. The sizes of the alleles are given in Table 11. There is no special  
15 preference for genotype with these alleles. Other alleles may occur at CSSM34 with different lengths of DNA.

In a seventh embodiment, the invention provides a method of identifying an animal of, or derived from, the Wagyu cattle breed with a high propensity for fat  
20 deposition in muscle, comprising the step of testing the animal for the presence or absence of an allele of the ETH10 DNA marker. Preferably the allele is allele 5. Allele 5 is 223 bp long.

In an eighth embodiment, the invention provides a  
25 method of identifying an animal of, or derived from, the Wagyu cattle breed with a low propensity for fat deposition in muscle, comprising the step of testing the animal for the presence or absence of an allele of the ETH10 DNA marker. Preferably the allele is allele 2. Allele 2 is  
30 217 bp long.

These embodiments of the invention are also applicable to the selection of animals for high or low fat content of milk respectively. The method is also useful for testing for fat levels in carcasses.

35 According to a second aspect the invention provides a method of detecting one or more of the alleles of the invention in an animal, comprising the steps of:



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a) obtaining a biological sample from the animal,

b) extracting DNA from the sample,

c) amplifying DNA from the relevant gene, and

5 d) identifying alleles in the amplified DNA.

Preferably the DNA is either of the 5' untranslated region of thyroglobulin or of DNA segments near the retinoic acid receptor gamma; if the animal is of the Wagyu breed of cattle, the DNA segments are near the  
10 retinol dehydrogenase 5 gene.

Preferably the biological sample is blood, but other biological samples from which DNA can be amplified may be used. For example hair root samples, cheek scrapings, skin samples and the like may be used.

15 Preferably for alleles of the 5' untranslated region of the thyroglobulin gene the region of DNA amplified includes a homopurine sequence and a copy of the monomeric dispersed repeat sequence. Preferably amplification is performed using polymerase chain reaction, but other DNA  
20 amplification methods such as ligase chain reaction are well known in the art, and may alternatively be used. Preferably the alleles are identified by polyacrylamide gel electrophoresis.

In a third aspect the invention provides  
25 oligonucleotide probes for amplification of the markers of the invention, selected from the group consisting of:

a) oligonucleotide probes for the 5' untranslated region of the thyroglobulin gene, having the sequences

30

TG5U2 5' ggg gat gac tac gag tat gac tg 3' (SEQ ID NO: 1)

TG5D1 5' gtg aaa atc ttg tgg agg ctg ta 3' (SEQ ID NO: 2)

b) oligonucleotide probes for amplification of  
35 the CSSM34 DNA marker, with the sequences

CSSM34U 5' cca taa ctc tgg gac ttt tcc tca 3' (SEQ ID NO. 6)

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CSSM34D 5' atg ttc agc cat ctc tcc ttg tcc 3' (SEQ ID NO. 7)

c) oligonucleotide probes for amplification of fragments from the RARG gene in cattle, with sequences

5

RARGSJ1U 5' cca agg atg cta atg aag atc ac 3' (SEQ ID NO: 9)

RARGSJ1D 5' gac taa cat tca tca aac acc gc 3' (SEQ ID NO 10)

RARGE3U1 5' ccg cga caa aaa ctg tat ca 3' (SEQ ID NO: 11)

RARGE3D1 5' ttg ctg acc ttg gtg atg ag 3' (SEQ ID NO: 12)

10 RARGE8U2 5' aat ccg aga gat gct gga ga 3' (SEQ ID NO: 13)

RARGE8D1 5' cac ccc tag aaa ctt tgg ca 3' (SEQ ID NO: 14)

d) oligonucleotide probes for amplification of fragments from the RDH5 gene in cattle, with sequences

15

RDH5U 5' atg cca agc tgc tct ggt t 3' (SEQ ID NO: 15)

RDH5D 5' tga agt gac tgt ttt atg cca cac 3' (SEQ ID NO: 16)

e) oligonucleotide probes for amplification of the ETH10 marker in Wagyu cattle, with sequences:

20

ETH10U 5' gtt cag gac tgg ccc tgc taa ca 3' (SEQ ID NO: 17)

ETH10D 5' cc tcc agc :ca ctt tct ctt ctc 3' (SEQ ID NO: 18)

25 In a fourth aspect the invention identifies Yeast Artificial Chromosomes, which are positive by hybridization to the oligonucleotide primers for CSSM34U and CSSM34D as well as for RARGE8U2 and RARGE8D1. These are 77D3, 77E3, 71G8, 94B4 and 71E4.

30 In a sixth aspect the invention provides an isolated nucleic acid molecule encoding part of the bovine retinoic acid receptor gamma, having the sequence set out in SEQ ID NO: 8 as defined herein.

35 The methods of the invention may be used both for the selection of breeding animals and for the selection of unpedigreed animals for entry into feed lots. In the latter case, the methods of the invention are applicable to

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deciding the length of time which animals spend in feedlots, since a high marbling score is unlikely to be attained with animals which are homozygous for allele 2 of the 5' untranslated region of thyroglobulin or allele 6 of CSSM34, or a Wagyu animal with allele 2 of ETH10, even after long feedlot holding.

The methods of the invention are applicable to animals including but not limited to cattle and other bovids, including water buffalo and bison, to other ungulates, including sheep, goats and deer, and to pigs.

For the purposes of this specification it will be clearly understood that the word "comprising" means "including but not limited to", and that the word "comprises" has a corresponding meaning.

#### Brief Description of the Figures

Figure 1 is a photograph of a single strand conformational polymorphism (SSCP) gel illustrating the polymorphism of the 5' untranslated region of the thyroglobulin gene.

Figure 2 shows the results of tests of associations between DNA markers on chromosome 5 and the marbling score.

#### Detailed Description of the Invention

The invention will now be described in detail by way of reference only to the figure and to the following non-limiting examples.

##### Example 1            CSSM66 is Associated with Marbling in Offspring of a Wagyu Sire

In the first experiment, DNA markers were selected from the bovine genetic linkage maps (Barendse et al, 1994, 1997; Bishop et al, 1994) so that a highly polymorphic DNA marker was present on each chromosome. These markers were evaluated for polymorphism on the Wagyu sire and if he was a homozygote an alternative marker was

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found. The resultant group of DNA markers were evaluated sequentially on the Wagyu offspring for linkage to marbling.

Since the sires and offspring were genotyped but  
5 no dams were genotyped, only those offspring that shared one allele with the sire provide direct information on linkage. The offspring that share none consistently were removed from the analysis as they indicate mispaternity. The offspring that share two alleles with the father can  
10 provide some information on linkage only if allele frequencies of the marker are known for this population. For these offspring the parental origin of each allele is uncertain, but probabilities of origin can be assigned for different genotypes of the dam, and the occurrence of the  
15 genotypes for the dams can be derived from the population frequencies of the alleles. These data are inferential, require a likelihood ratio approach for analysis, and were not used. Clearly, the more alleles to the marker the more information on linkage is available for analysis, since the  
20 offspring is more likely to share only one allele with the sire.

The results were analysed by segregating the individuals by marbling score and by paternal allele after parentage testing had been completed. These 2 x 2 tables  
25 were analysed via contingency chi-square analyses to test associations that are not dependent upon a genetic model. They were also analysed by setting expected proportions equal, as if there was a single Mendelian locus on that particular chromosome with an additive effect on marbling.

30 The fingerprinting of the offspring of the Wagyu sire showed 5 offspring that regularly failed to share a band with the sire, and so they were excluded from further analyses, although the samples were retained since they provided clear landmarks on the autoradiograms. These  
35 results are summarized in Table 1.

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Table 1

Association Between the DNA Marker CSSM66  
and the Marbling Score Among Offspring of the Wagyu Sire

M2	M4	Allele
49	7	2
37	26	4

5

$$\chi^2_1 = 12.24 \text{ } p < 0.001$$

M2 and M4 are marbling scores of 2 and 4 respectively.  
Allele is the allele of the sire inherited by the steer.

10 Alleles are ranked in mobility, with the fastest migrating  
allele = 1

The polymorphic DNA marker CSSM66 showed an  
association to marbling score in the offspring of the Wagyu  
15 sire, with a probability of less than 0.001 of this  
occurring by chance. This marker was the 12th in a series  
of loci chosen at random. The locus RM180 was tested, and  
found to show a non-significant deviation from the expected  
values. RM180 is 18 cM distal to CSSM66, indicating that a  
20 gene affecting marbling would be in the close vicinity of  
CSSM66.

Example 2                      CSSM66 and Marbling in Angus and Shorthorn  
Offspring

25                      The DNA markers that showed a positive  
association in the first experiment were tested in the  
second experiment. They have an *a priori* expectation of  
being positively associated, and a lower threshold for  
significance is acceptable. Two approaches were taken to  
30 these data. In the first, the two groups of extreme  
marbling scores were compared irrespective of ancestry.  
This rough analysis would show an association if there were  
linkage disequilibrium between the DNA markers and a locus  
that affects the marbling score. Irrespective of the prior

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linkage demonstrated for these regions, however, these results could be biased if they are dominated by a single sire that contributed many individuals of one particular marbling score, where this sire was a homozygote for the DNA marker. In the second approach, only those animals that were drawn at random and were essentially unrelated to others in the study were analysed by marbling groups and by genotype for a population association. For the animals in sire groups, only those from sires that had offspring of low and of high marbling score were retained and the rest excluded. The gene frequencies of the two groups were compared via the chi-square analysis. The relative risk was calculated via the method of Woolf (1955).

CSSM66 was tested over the Angus and Shorthorn offspring irrespective of ancestry, and the results are shown in Table 2.

Table 2

Association Between the DNA Marker CSSM66  
and Marbling Score Among Angus and Shorthorn Steers

M1	M4	Allele
22	20	1
11	11	2
25	28	3
14	39	4
5	5	5
57	52	6
2	2	7

$$\chi^2_6 = 5.82 \text{ } p < 0.45 \text{ n.s.}$$

M1 and M4 are marbling scores of 1 and 4 respectively. Allele is the allele of the steer. Alleles are ranked in mobility with the fastest migrating allele = 1, and are comparable to Table 1.

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No significant association was found between CSSM66 and marbling score. The allele '4', which had been found linked to high marbling scores in the Wagyu experiment, was twice as common in animals of high marbling but there is no corresponding allele that showed an excess among animals with low marbling.

RM180 showed no association.

Example 3                      Identification of Thyroglobulin

10                      Polymorphism Associated with Marbling

Primers were designed so as to be complementary to the 5' untranslated region (5'UTR) of the thyroglobulin gene (TG: Genbank accession X05380). This sequence contains a homopurine sequence and a copy of the bovine monomeric dispersed repeat (de Martynoff et al, 1987), and the primers were located to include both of these features. The primer sequences are :

TG5U2    5' ggg gat gac tac gag tat gac tg 3' (SEQ ID NO: 1)  
20    TG5D1    5' gtg aaa atc ttg tgg agg ctg ta 3' (SEQ ID NO: 2)

and the expected size of the fragment is 545 base pairs. The fragment was amplified by the polymerase chain reaction (PCR), and tested for polymorphism by single strand conformational analysis (SSCA) using previously described methods (Mullis et al, 1986; Orita et al, 1989; Barendse et al, 1993). The fragments were amplified with an annealing temperature of 55°C at 2 mM magnesium chloride for at least 30 cycles of the PCR. The fragments were then separated for 22 hours on 0.4 mm gels composed of 8% acrylamide (89:1::acrylamide:bis-acrylamide), 0% glycerol, 0.5 x TBE (1 x TBE is 0.089 M TrisHCl, 0.089 M boric acid, 0.002 M disodium ethylenediaminetetraacetic acid) in 38 cm wide x 50 cm long gels at 3 Watts at room temperature. These conditions provide the best means of separating all three alleles, particularly the rare 1 allele, at this locus, although several different

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conditions of glycerol (5 and 10 percent) and power (5 and 7 W) provide separation of the alleles 2 and 3. The fragments were detected by autoradiography.

The primers for the 5'UTR of thyroglobulin produce a single fragment, which shows three alleles when run on single strand conformational polymorphism (SSCP) gels, as illustrated in Figure 1. There are 11 complete genotypes on the gel. The top series of bands is one conformation of the DNA fragment and is uninformative. The bottom series of bands is the alternative conformation which shows three alleles. The genotypes are in the order:

33 22 23 23 22 22 23 22 22 23 13

Five associations were calculated. The first was for all individuals that were sampled at random, as summarized in Table 3. The probability of the association occurring by chance is less than 0.05, with allele 3 being associated with high marbling levels. The relative risk of possessing allele 3 is 3.81.

Table 3

Association Between Thyroglobulin and  
Marbling Score Among the Angus and  
Shorthorn Steers Drawn from the Cattle Population

Marbling	Genotypes		
	22	23	33
M1/2	10	7	0
M4/5	6	15	1

$$\chi^2_1 = 3.94 \text{ } p < 0.05$$

M1/2 are low marbling scores and M4/5 are high marbling scores. Genotype is the genotype of the steer. Allele 1



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is extremely rare, and only 2 copies of this allele have been seen in 264 individuals.

The one genotype of '33' was merged with the '23' genotypes for the M4/5 class to calculate the chi-square. The relative risk for the '3' allele and increased marbling is 3.81.

In the second association, the steers compared were derived from sires who produced steers of high and of low marbling, and again there is a small sample size. The results are summarized in Table 4. This association shows the same direction, where allele three is associated with high marbling scores, and has a probability less than 0.05 of occurring by chance.

Table 4

The Association Between Thyroglobulin and Marbling Score Among the Angus and Shorthorn Steers Drawn from Families Where the Sire had Offspring of High and of Low Marbling Score

M1/2	M4/5	Allele
60	45	2
22	33	3

$$\chi^2_1 = 4.25 \text{ } p < 0.04$$

M1/2 are low marbling scores and M4/5 are high marbling scores. Allele '1' is extremely rare and only 2 copies of this allele have been seen in 264 individuals.

Example 4      DNA Sequence of the Thyroglobulin Alleles  
TG5U2 and TG5D1 Described in Example 3

The DNA sequence of the thyroglobulin gene, amplified by the primers TG5U2 and TG5D1 described in Example 3, shows three alleles in the study population.

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These alleles were isolated, and the DNA sequence of each was determined using the standard dideoxy sequencing method (Sanger et al, 1977). The numbering of the alleles corresponds to that in Figure 1. The DNA sequence of each

5 allele is given in Table 5, and the DNA sequence differences responsible for the variation are highlighted.

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Table 5

The Sequences of Three of the  
Alleles Amplified by the TG5U2 and TG5D1 Primers.

- 5 The sequence differences that define the alleles are in  
bold capital letters.

Allele 1 (SEQ ID NO: 3)

10 ggggatgactacgagtatgactgtgCGTGTGTTTGGCTTATCTCATCAAAATCTCTACA  
ttctgtgttaatggatctgcctgttttGTTCCCTGCCATATCCTCATGGCCTAGAATAG  
tgtctgcttctctatcagactCTAAAGAAACATTGCTAGGAGGGAAGGAAGGAGCATGG  
atgaggagggagggagcattgtgtttctctCACGGTGGGCCTGAACGTGTGGCCACCA  
agttgttaactttggcctttaccctgaagatgaattatgaagccacacccccagttct  
15 tccttggTGGCTCAGATGGTCAAGAAATCCACCTGCAATGCGGGAGACCTGGGTTTGATC  
cctgggttgggaagatCCcctggagaagggaatggctaccactccagtattctggcct  
ggagaatcccatggacagaggagcctggcgggatgcagtccatggggTctcagagagtc  
agatgtgactgagcgactttcacacacaCTcgtccctggttctgctcccctacagcctc  
cacaagattttcac

20

Allele 2 (SEQ ID NO: 4)

ggggatgactacgagtatgactgtgCGTGTGTTTGGCTTATCTCATCAAAATCTCTACA  
ttctgtgttaatggatctgcctgttttGTTCCCTGCCATATCCTCATGGCCTAGAATAG  
25 tgtctgcttctctatcagactCTAAAGAAACATTGCTAGGAGGGAAGGAAGGAGCATGG  
atgaggagggagggagcattgtgtttctctCACGGTGGGCCTGAACGTGTGGCCACCA  
agttgttaactttggcctttaccctgaagatgaattatgaagccacacccccagttct  
tccttggTGGCTCAGATGGTCAAGAAATCCACCTGCAATGCGGGAGACCTGGGTTTGATC  
cctgggttgggaagatCCcctggagaagggaatggctaccactccagtattctggcct  
30 ggagaatcccatggacagaggagcctggcgggatgcagtccatggggTctcagagagtc  
agatgtgactgagcgactttcacacacaTtcgtccctggttctgctcccctacagcctc  
cacaagattttcac

35

- 18 -

Allele 3 (SEQ ID NO: 5)

ggggatgactacgagtatgactgtgcgtgtgtttggcttatctcatcaaaatctctaca  
 ttctgtgttaatggatctgcctgttttgttccctgccatatcctcatggcctagaatag  
 5 tgtctgcttctctatcagactctaaagaaacattgctaggagggaaggaaggagcatgg  
 atgaggaggggaggagcattgtgtttctctcacgggtgggcctgaacgtgtggcccacca  
 agttgttaactttggcctttaccctgaagatgaattatgaagccacacccccagttct  
 tccttgggtggctcagatgggtcaagaatccacctgcaatgcgaggagacctgggtttgatc  
 cctgggttgggaagatTccctggagaagggaatggctaccactccagttattctggcct  
 10 ggagaatcccatggacagaggagcctggcgggatgcagtcctatgggtctcagagagtc  
 agatgtgactgagcgactttcacacacaTtcgtccctgggttctgctccctacagcctc  
 cacaagattttcac

Example 5                      Thyroglobulin Polymorphism in Wagyu  
 15                                      Offspring

For the thyroglobulin polymorphism, the offspring  
 of the Wagyu sire were analysed retrospectively to  
 determine whether there was an association to marbling  
 score and whether this association was in the same general  
 20 direction as that found in the Angus and Shorthorn steers.  
 Since the Wagyu samples were collected from three different  
 feedlots at three different times, these samples were  
 analysed separately. Furthermore, since there are only  
 effectively two alleles at the thyroglobulin polymorphism  
 25 (see below), this locus was analysed for population  
 association rather than genetic linkage, using goodness of  
 fit contingency chi-squares, since the allelic contribution  
 of the sire cannot be ascertained in the heterozygotes as  
 the maternal genotypes are not available. In two of the  
 30 three Wagyu subsamples there were insufficient individuals  
 with extreme marbling scores, so all the marbling scores  
 were analysed.

The probabilities (P) of the independent chi-  
 squares were transformed using natural logarithms and  
 35 summed (Sokal and Rohlf, 1981) to form a combined  
 probability estimate for the association between  
 thyroglobulin and marbling. The value of  $-2\sum \ln P$  is

- 19 -

distributed as a chi-square with the number of degrees of freedom equal to twice the number of component probabilities.

The third, fourth and fifth associations were tested among the Wagyu offspring. Two of these three associations have probability values less than 0.05 of occurring by chance when the genetic model is assumed to be dominant inheritance; having one copy of the '3' allele gives the same effect as having two copies of the '3' allele. None of the associations has a probability level below 0.05 when a codominant model is assumed. Of these three associations, one uses the extremes of marbling, as shown in Table 6.

Table 6

The First Sample of Wagyu Steers that are Extreme for Marbling Genotyped for the Thyroglobulin Polymorphism.

Genotype	Marbling	
	M2	M4
22	44	5
23	33	12
33	6	2

$\chi^2_1 = 4.41$   $p < 0.04$  (A: Dominant mode)  
 $\chi^2_2 = 4.43$   $p < 0.11$  (B: Co-dominant mode)

Two genetic models are used. Model 1 assumes a dominant mode of inheritance, and model 2 assumes a co-dominant mode of inheritance.

Note: The 33 genotypes were added to the 23 genotypes to get the dominant mode.

The thyroglobulin genotypes were compared to the marbling scores, and an association between higher marbling

- 20 -

score and the possession of one or more copies of the '3' allele was formed, with a probability less than 0.05 of occurring by chance. For the two other associations the thyroglobulin genotypes were compared to all the marbling scores, since these subsamples had insufficient numbers of animals with extreme marbling scores for statistical significance to be demonstrated. These results are summarized in Tables 7 and 8.

10

Table 7

The Second Sample of the Wagyu Steers:  
Analysis of the Trend to Higher Marbling Score  
Among Individuals of the 23 Genotype

Genotype	Marbling Score				
	M2	M3	M4	M5	M6
22	10	16	6	3	0
23	14	23	23	7	2
33	3	4	5	2	0

15

$$\chi^2_1 = 4.20 \text{ } p < 0.05 \quad (\text{A: } 22 \text{ vs } 23/33)$$

$$\chi^2_2 = 4.68 \text{ } p < 0.10 \quad (\text{B: Co-dominant})$$

20

There were insufficient animals of extreme marbling score to analyse only the extremes.

Instead of extremes being compared, M2 plus M3 is compared to M4, M5 plus M6.

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Table 8

The Third Sample of the Wagyu Steers:  
Analysis of the Trend to Higher Marbling Score  
Among Individuals of the 23 Genotype

5

Genotype	Marbling Score				
	M2	M3	M4	M5	M6
22	4	28	5	1	0
23	11	48	7	1	0
33	0	5	1	1	0

$$\chi^2_1 = 0.11 \text{ } p < 0.75 \quad (\text{A: } 22 \text{ vs } 23/33)$$

$$\chi^2_2 = 1.54 \text{ } p < 0.47 \quad (\text{B: } \text{Co-dominant})$$

- 10 There were insufficient animals of extreme marbling score to analyse only the extremes.

Instead of extremes being compared, M2 plus M3 is compared to M4, M5 plus M6.

15

- The numbers of individuals with marbling scores M2 and M3 were combined and compared to the combined number for marbling scores of M4, M5 and M6. One of the two samples showed an association between possession of one or more copies of the '3' allele and higher marbling scores (Table 7), with a probability less than 0.05 of occurring by chance. The other sample (Table 8) showed no association of thyroglobulin with marbling score. In no case was there an association between possession of the '22' genotype and high marbling score.

25

The probabilities for the five thyroglobulin tests were summed in two ways as three of the five tests having two models - dominant and co-dominant. The results are shown in Table 9.

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Table 9

The Combination of Chi-Square Probabilities for all the Associations Between Thyroglobulin and Marbling

	Chi-Square		P	lnP
1.	$\chi^2_1 = 3.94$		p = 0.047	-3.058
2.	$\chi^2_1 = 4.25$		p = 0.039	-3.244
3.	$\chi^2_1 = 4.41$	A	p = 0.036	-3.324
	$\chi^2_2 = 4.43$	B	p = 0.109	-2.216
4.	$\chi^2_1 = 4.20$	A	p = 0.040	-3.219
	$\chi^2_2 = 4.68$	B	p = 0.096	-2.216
5.	$\chi^2_1 = 0.11$	A	p = 0.745	-0.294
	$\chi^2_2 = 1.54$	B	p = 0.464	-0.768

5

$$\chi^2_5 = 26.278 \quad p < 0.005 \text{ (A)}$$

$$\chi^2_5 = 23.258 \quad p < 0.005 \text{ (B)}$$

10 All associations bar one show an association between the 3 allele and high marbling score, one test showing no association. The A series represents the dominant mode of inheritance, while the B series represents the co-dominant mode.

15 The two combinations are thus all the dominant models and all the co-dominant models. Both of these summations have probabilities less than 0.005 of occurring by chance, and are extremely significant.

20 The Wagyu sire is a heterozygote for this polymorphism, with the genotype '23'. Among the 335 offspring of the Wagyu sire tested none showed the '1' allele.

Example 6Chromosome 5 is Associated with Marbling in Offspring of a Wagyu Sire

25 Surprisingly also, significant associations to marbling score were found with the anonymous DNA markers CSSM34 and ETH10 and these will be described in the next



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several examples. These markers had been assigned to bovine chromosome 5 on the International Bovine Reference Family Panel (described in Barendse *et al*, 1997), with a location about one third of the way down the chromosome.

5 Using the Wagyu family material described in Example 1 above, DNA markers from chromosome 5 were genotyped on the Wagyu sire and his offspring. The DNA marker from chromosome 5 with the best association in the 2 x 2 contingency chi-square is ETH10 (Toldo *et al*, 1993), as

10 shown in Table 10.

Table 10

Association Between the DNA Marker ETH10 and the Marbling Score Among Offspring of the Wagyu Sire

15

M2	M4	Allele
60	12	2
24	17	5
$\chi^2_1 = 8.42$	$P < 0.005$	
$\chi^2_1 = 16.28$	$P < 0.0001$	(Dominant model)

20

M2 and M4 are marbling scores of 2 and 4 respectively. Allele is the allele of the sire and that

25 the steer inherited. Alleles are ranked in mobility, with the fastest migrating allele = 1. Allele 2 is 217 bp long and allele 5 is 223 bp long. Other lengths of alleles are expected at the ETH10 DNA marker.

The polymorphic DNA marker ETH10 showed an

30 association to marbling score in the offspring of the Wagyu sire, with a probability of less than 0.005 of this occurring by chance, and with a probability of less than 0.0001 of this occurring by chance if a dominant mode of inheritance is assumed. This marker was the tenth in a

35 series of loci chosen at random. This association indicated that a gene affecting marbling would be in the close vicinity of ETH10. Allele 5 of ETH10 was associated

- 24 -

with higher marbling scores, while allele 2 was associated with lower marbling scores. The marker CSSM34 showed no association with marbling in this family: the sire provided informative meioses, but there was no evidence for  
5 segregation of marbling near CSSM34.

Example 7                      Chromosome 5 Markers and Marbling in Angus and Shorthorn Steers

A series of DNA markers from chromosome 5 which  
10 are located on either side of ETH10 were tested on a sample of Angus and Shorthorn steers of known ancestry. These are the same steers as those used in Example 2. The analysis was performed so that at most two steers from each  
15 grandsire were used. Only steers of extreme marbling score were used, so that a comparison of extreme marbling scores was made across a cross-section of the beef industry. The DNA marker CSSM34 (Moore et al, 1994) had the most  
20 significant association with marbling, as shown in Table 11, and the pattern of association of marbling to the DNA markers shows that a marbling gene on chromosome 5 is located in close proximity to CSSM34, as shown in Figure 2. ETH10 showed no association to marbling in these Angus and Shorthorn steers.

25                                      Table 11  
Association Between the DNA Marker CSSM34  
and the Marbling Score Among Offspring  
of Known Angus and Shorthorn Sires

	ALLELE					
	1	2	3	4	5	6
MARBLING						
M1	3	31	14	5	18	9
M4+	1	49	24	5	7	2

30

$$\chi^2_5 = 16.63 \quad P < 0.0053$$

- 25 -

M1 and M4+ are marbling scores of 1 and greater than or equal to 4 respectively. Allele is the allele that the steer possesses. Alleles are ranked in mobility, with the fastest migrating allele = 1. The alleles differ in size, hence the differences in mobility, and allele 1 = 100 bp (base pairs), allele 2 = 102 bp, allele 3 = 106 bp, allele 4 = 108 bp, allele 5 = 110 bp and allele 6 = 112 bp. Other alleles with different sizes are expected to exist.

The association between marbling and CSSM34 is strong, and has a probability of less than 0.0053 of occurring by chance. Since a marbling gene had already been demonstrated through the association with ETH10 in Example 6, this association provides strong evidence of the existence of a gene affecting marbling score on chromosome 5. No other DNA marker tested for chromosome 5 had as strong an association with marbling. The next best association was to the gene LALBA, but that had a probability only slightly less than 0.05 of occurring by chance. Since LALBA has a genetic distance of approximately 2 cM from CSSM34 (Barendse et al, 1997), this indicates that CSSM34 is in close allelic association with a marbling gene.

Several of the alleles of CSSM34 show allelic association with marbling. Notably alleles 2 and 3 are associated more with higher marbling scores, while 5 and 6 are associated with lower marbling scores.

Example 8                      CSSM34 Evaluated on Randomly Drawn Angus and Shorthorn Steers of Unknown Ancestry

CSSM34 was then tested on randomly-collected Angus and Shorthorn steers of unknown ancestry. Firstly, a positive association would confirm the results found in steers of known ancestry. If the results showed the same pattern of allelic disequilibrium this would indicate that the marker CSSM34 was not only a robust predictor of marbling capacity, but that it was extremely closely

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associated with the causal mutation for marbling.

Secondly, a positive association would indicate that the marker CSSM34 could be used as a tool in feedlots to draft animals into particular feeding regimes on the basis of  
5 their genotype, and in that way alter the probability of achieving desired marbling scores.

The sample of cattle for this experiment was obtained by bleeding 50 to 100 cattle each week of the Angus or Shorthorn breed of unknown parentage from the same  
10 abbatoir. In addition to the breed identification, the identity of the vendor was recorded as well as the standard chiller room and feedlot data such as marbling score, subcutaneous fat thickness, age, feeding regime and carcass weight. By sampling each week and by maximizing the number  
15 of vendors that were present in a sample, a wide cross section of the beef industry was obtained. There are an average of five steers per vendor in the sample with a total of 162 vendors. DNA was extracted from all available blood samples. The data form a contingency table with  
20 marbling scores as the rows and the allele possessed by the individual as the columns. The contingency data were analysed using the G statistic (Sokal and Rohlf, 1981), since some of the cells had small expected numbers and the G statistic provides a superior approximation to the chi-  
25 square distribution.

The association between CSSM34 and marbling in the randomly-collected Angus and Shorthorn steers is shown in Table 12.

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Table 12

Association Between CSSM34 and Marbling Scores  
for Randomly Collected Angus and Shorthorn  
Steers of Unknown Ancestry

5

MARBLING	ALLELE					
	1	2	3	4	5	6
M1	4	35	35	11	8	9
M2	8	257	261	47	89	66
M3	4	213	158	24	41	33
M4	2	80	58	11	16	7
M5	0	13	8	1	2	0

$G_{adj} = 32.17$        $df\ 20$        $P < 0.05$

M1 to M5 are marbling scores of 1 through to 5.

10 Allele is the allele that the steer possesses. Alleles are ranked in mobility, with the fastest migrating allele = 1, and are the same designations as in Table 11.  $G_{adj}$  is the G statistic adjusted using the Williams correction (Sokal and Rohlf, 1981).

15 This comparison shows that there is clearly an allelic association between marbling score and allele at the DNA marker CSSM34, and is consistent with the previous analyses. Since the animals are sampled at random from the population and their ancestry is unknown, our result  
20 confirms that this marker can predict average marbling score without knowing the ancestry of a steer. When allele 2 is compared to allele 6 from Table 12 the  $G_{adj} = 15.21$ ,  $df\ 4$ ,  $P < 0.005$ , indicating that there is a highly significant difference in marbling score for those with  
25 allele 2 compared to those with allele 6. This association between alleles 2 and 6 and marbling is consistent with the previous sample, shown in Table 11, which indicates that the allelic association is not only consistent but is also stable. Such associations occur

when the polymorphism is responsible, or where the marker is closely associated with the responsible gene.

Example 9                    CSSM34 is Closely Associated with the  
5                                Retinoic Acid Receptor Gamma (RARG) Gene  
                                 While ETH10 is Closely Associated with the  
                                 Retinol Dehydrogenase 5 (RDH5) Gene

No immediate candidate genes for marbling were evident, although several adipocyte differentiating factors  
10 were expected to be on chromosome 5 on the basis of their genomic locations in humans and mice. CSSM34 is located very close to collagen 2 alpha 1 (COL2A1), but that gene is not a candidate gene for marbling. Based on the human map near COL2A1, two candidate genes suggested themselves.  
15 These are the genes for retinoic acid receptor gamma (RARG) and 11-cis and 9-cis retinol dehydrogenase (RDH5). RARG is a nuclear receptor for all-trans retinoic acid, which is derived from retinol, and RDH5 catalyses the interconversion of 11-cis and 9-cis retinol to 11-cis and  
20 9-cis retinoic acid (Mertz et al, 1997). The level of retinol (vitamin A) in the blood is linearly related to marbling score (Torii et al, 1996), and the retinoic acid receptors are known factors in the differentiation of pre-adipocytes (Ailhaud et al, 1992; Darimont et al, 1993; Smas  
25 and Sul, 1995). Importantly, the concentration of retinol has an impact on marbling score but no impact on the thickness of subcutaneous fat, suggesting that DNA tests for this region could identify a propensity for increase marbling without necessarily also increased fat levels in  
30 other fat depots of cattle. A standard treatment in Japan to enhance marbling score is to reduce the level of vitamin A precursors such as  $\beta$ -carotene in the diet of the steer. RDH5 has been sequenced in cattle, but RARG had not previously been sequenced and a DNA clone had not been  
35 isolated.

We sought to identify the genes with which the DNA markers CSSM34 and ETH10 were associated. The first

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stage was to identify fragments from the bovine RARG and RDH5 genes and locate these on the bovine chromosomes at high resolution relative to the CSSM34 and ETH10 polymorphisms. A whole-genome radiation hybrid panel (Womack et al, 1997) was used to locate CSSM34 and ETH10 relative to several genes and DNA markers from chromosome 5. RDH5 proved to be close to ETH10, at a distance of 1.01 centi-Rads (cR). RARG proved to be close to CSSM34, at a distance of 3.25 cR. These distances represent extremely close physical distances, and these DNA markers are clearly closely associated with the respective genes. The primers for CSSM34 and for RARG were then used to probe a Yeast Artificial Chromosome (YAC) library, and every DNA clone that was positive for CSSM34 was also positive for RARG. We have thus identified cloned DNA fragments for the cattle RARG gene, and all of these contain the CSSM34 DNA marker. A RARG-associated polymorphism, CSSM34, can be used to predict marbling score in, but not limited to, the Angus and Shorthorn breeds of cattle. Further, a RDH5 associated polymorphism, ETH10, is linked to marbling in the Wagyu breed of cattle.

The bovine sequence for RDH5 (Genbank accession X82262, Simon et al, 1995) was used to design primers for RDH5. The primers RDH5U and RDH5D generate a 282 bp fragment from bovine DNA. This fragment is polymorphic in cattle, with two alleles.

No bovine sequence for RARG has been described, so the human and mouse sequences were used to generate fragments from bovine DNA. The human sequence for RARG (Genbank accession M38258, Lehmann et al, 1991) and the mouse sequence for RARG (Genbank accession M34476, Giguere et al, 1990) were obtained to design heterologous primers for RARG. Primers were used to amplify the intron between exon 6 and exon 7, and the amplified fragment was cloned and sequenced using standard dideoxy sequencing methods (Sanger et al, 1977). The fragment was analysed using fluorescent labelling via the ABI cycle sequencing protocol

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(Perkin Elmer, Foster City, California, USA) to confirm that RARG was cloned in cattle. The sequence is shown in Table 13. Primers (RARGSJ1U, RARGSJ1D) derived from this sequence amplify bovine DNA. Other primers were also  
5 designed to amplify RARG from bovine DNA (RARGE3U1, RARGE3D1 and RARGE8U2, RARGE8D1). The sequences of these primers are shown in Table 14, together with the sequences for primers RDH5U and RDH5D for RDH5, and primers ETH10U and ETH10D for ETH10.

10

Table 13

The DNA Sequence for the  
Cloned Fragment of Cattle RARG

15 (SEQ ID NO: 8)

tatgatacnaattcgagctcggtacctacatgttcccaaggatgctaataagatcact  
gacctccggggcatcagcaccaaggggttagtcgggagcaagcctcccctctgtcttctc  
ggagctgccggtctccaggtcaggcagagacaagagcanagtgggggtataatcaggca  
20 gcctgcactcgcacctcgtccgctgcatgctagtgggaacacttgggtgaaaaatacc  
tttcctttttgtaccttggttttctggttgtaggagtgaaacaagttaacacacaacag  
gcctacagctgtgctgagttataaagttcagtgccctcctgccctggatggagcagatgt  
ttccancatcacaggaangttgattggacgcctggcacgcggtgtttgatgaatgtta  
gtcntagtgataaatgttattaagaacagccatgggcttacggaggggtccanngtgtg  
25 tggctggaagtgggcgctgtgtgatcttggaggagacagcctgaaagaaagtgggcagt  
ggacttggcagagaagacaggcagagttccaggcagaggagtgggcccaggagcttta  
cagtagaaagagggagagaaagaagcagacagagataacaggcctgtgatgggagcccc  
agagggcagtcagcagagttagggaggccgctaggtgctgtacntcagccccctga  
actcttggttcntccactgcaggagcagaaagggccattaccctgaagatggagattcca  
30 ggcccgatgcctcccctgatccgagaaatgctggagaaccccgaaatgtttgaggacga  
ctcctcgcagcctggccctcaccccaaggcctctagcgaggatgaggttccctggggatc  
ctctagagtcgacctgcaggcatgcaagctaggcactggccgctcgttttacaacaa



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Table 14

Oligonucleotide Primers for Amplification of  
DNA Encoding RARG

5	RARGSJ1U	5'	cca agg atg cta atg aag atc ac 3'	(SEQ ID NO: 9)
	RARGSJ1D	5'	gac taa cat tca tca aac acc gc 3'	(SEQ ID NO 10)
	RARGE3U1	5'	ccg cga caa aaa ctg tat ca 3'	(SEQ ID NO: 11)
	RARGE3D1	5'	ttg ctg acc ttg gtg atg ag 3'	(SEQ ID NO: 12)
	RARGE8U2	5'	aat ccg aga gat gct gga ga 3'	(SEQ ID NO: 13)
10	RARGE8D1	5'	cac ccc tag aaa ctt tgg ca 3'	(SEQ ID NO: 14)
	RDH5U	5'	atg cca agc tgc tct ggt t 3'	(SEQ ID NO: 15)
	RDH5D	5'	tga agt gac tgt ttt atg cca cac 3'	(SEQ ID NO: 16)
	ETH10U	5'	gtt cag gac tgg ccc tgc taa ca 3'	(SEQ ID NO: 17)
15	ETH10D	5'	cc tcc agc cca ctt tct ctt ctc 3'	(SEQ ID NO: 18)

The loci CSSM34, RARG (primers RARGSJ1U and  
20 RARGSJ1D) and RDH5 as well as LALBA, ETH10 and CSSM22  
(Moore et al, 1994) were genotyped on the whole genome  
radiation panel of Womack and associates (1997). The  
results of these genotypes are shown in Table 15.

Table 15

The Genotypes for the 6 loci LALBA, CSSM34, RARGSJ1, ETH10, CSSM22 and RDH5 from the Whole Genome Radiation Hybrid Panel of Cattle

[illegible]

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The 0 and 1 symbols represent presence or absence of the locus in a particular radiation hybrid clone. The closer two loci are, the more hybrid clones they have in common, and the fewer the differences between them.

5           The hybrid clone data place RARGSJ1 between LALBA and CSSM34, with a 3.25 cR distance between RARGSJ1 and CSSM34. This is equivalent to a few hundred kilobase pairs between the amplified DNA fragments. These data also place RDH5 1.01 cR from ETH10. Alternatively, CSSM34 is 54 cR  
10 from RDH5, a substantial physical distance.

          The small relative distance between CSSM34 and RARGSJ1 indicates that both DNA fragments may be contained on a single DNA clone. To test this proposition, a YAC (Yeast Artificial Chromosome) library was screened by  
15 hybridization with both of the primers for CSSM34 after these primers were end-labelled with  $^{32}\text{P}$   $\gamma$  ATP using Polynucleotide Kinase (Richardson, 1981). The yeast library is contained in the yeast strain AB1380 with the bovine DNA contained in the vector pYAC4. This library was  
20 constructed using the methods of Libert and coworkers (1993), and has been deposited in the Resource Centre of the German Human Genome Project (<http://web.rzpd.de/index.html>). Positive clones were identified by autoradiography. The library was also screened with both primers for RARGE8  
25 after the primers were end-labelled with  $^{32}\text{P}$   $\gamma$  ATP. Positive clones were identified by autoradiography, and all positive clones were the same as the positives for CSSM34. The clone names are 77D3, 77E3, 71G8, 94B4 and 71E4. This demonstrates that CSSM34 is closely associated with the  
30 genomic sequence of RARG.

#### DISCUSSION

          The linkage between CSSM66 and marbling strongly suggests that a locus affecting marbling is located on  
35 bovine chromosome 14. The lack of association to RM180 indicates that candidate genes for the effect will be located close to CSSM66, which is located to the proximal

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third of the chromosome (Barendse *et al*, 1997). The lack of a gross association between alleles of CSSM66 and marbling indicates that the causative gene is not very close to CSSM66. The gene for thyroglobulin is located  
5 some 7 cM from CSSM66, and this gene shows an extremely significant association with marbling score, consistent with a marbling gene on chromosome 14.

Thyroglobulin is encoded by a massive 300 kilobase stretch of genomic DNA. This protein acts as  
10 the molecular store for triiodothyronine and tetraiodothyronine (Parma *et al*, 1987), hormones that are known to have an effect on adipocyte differentiation. It has been known for a half a century (Salter, 1950) that the thyroid hormones are associated with the deposition of fat  
15 cells in muscle. Recent experiments in cell culture have shown the role of these hormones in the growth and differentiation of adipocytes (Levacher *et al*, 1984; Darimont *et al*, 1993). Furthermore, structural mutational variation in the thyroglobulin gene has been shown to be  
20 causally implicated in congenital goitre in Afrikaner cattle (Ricketts *et al*, 1985), so it is unlikely that structural mutations at this gene would be responsible for variation in fat cell differentiation. In addition, the thyroglobulin genomic DNA sequence is unusually low in  
25 variation both in humans and cattle (Baas *et al*, 1984; Georges *et al*, 1987), suggesting tight control through natural selection. Alterations in the processing of iodine are likely to have catastrophic results, as can be seen when the diet of humans is deficient in iodine, an element  
30 critical to the production of thyroid hormones. The consequences of this deficiency are cretinism, failure of proper development and growth of the bones resulting in a high body weight to length ratio, and myxoedema. The level of the thyroid hormones is implicated in adipocyte  
35 differentiation and has an effect on metabolic rate, which in turn has an impact upon the amount of energy available for storage.

- 35 -

Since the 5' untranslated regions (5'UTR) of genes are critical in transcription and translation (Ptashne, 1988, Kozak, 1991), and hence affect the level and availability of a protein, a DNA polymorphism was sought in the 5'UTR of thyroglobulin. The novel polymorphism identified in this specification shows an association to marbling which has a consistent direction, in which allele '3' is associated with higher marbling scores in four of five subdivisions of the data. For a small sample of unrelated animals the relative risk of allele '3' compared to allele '2' was 3.81; thus animals with high marbling score are almost 4 times more likely to have at least one copy of allele '3' than to be a '22' homozygote. Consistent with this model, the Wagyu sire is a '23' heterozygote and segregates marbling score on chr. 14. It would have been a powerful test of this fragment had he been a homozygote. The overall probability level for the association between marbling score and this thyroglobulin polymorphism is very strong, being less than 0.005, and the evidence for marbling gene on chromosome 14 is convincing, being probability level for the association less than 0.0001 irrespective of the mode of inheritance.

Due to linkage disequilibrium, polymorphisms located near to thyroglobulin on chromosome 14 will also have some predictive value for marbling. However, CSSM66 is not one of them, consistent with the 7 cM distance between CSSM66 and thyroglobulin; linkage disequilibrium is usually expected when the genetic distance is low, generally when it is less than 3 cM. Nevertheless, unless it is proved that there are other likely genes affecting marbling in this region of chromosome 14 it must be assumed that these other polymorphisms are predicting the same test described in this report. Polymorphisms in the 5'UTR of the thyroglobulin molecule of other mammalian species may predict levels of fat in those species, since the action of the iodothyronines is conserved across species, and the structure of thyroglobulin is relatively strongly

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conserved. There is 84% homology of sequence between humans and cattle, and 75% homology between mice and cattle.

Obviously, marbling is affected by the products  
5 of several genes as well as being subjected to  
environmental influences, so one genetic test will not  
cover all the variation. Thus, some '33' homozygotes are  
expected to have low marbling scores due to the influence  
of variation at other genes or of suboptimal management.  
10 Nevertheless, selection of animals on the basis of the  
thyroglobulin polymorphism described here will shift the  
proportions of animals that show high and low levels of  
marbling, either in feedlots or when selecting parents to  
generate steers.

15 CSSM66 has been shown to be linked to milk fat  
percentage in USA Holstein dairy cattle (Ron *et al*, 1996),  
so it is expected that the polymorphism in the  
thyroglobulin gene described here will be predictive in the  
selection of cattle for high levels of milk fat, since the  
20 thyroid hormones are known to have an impact on the fat  
percentage of milk (Folley and Malpress, 1948).

The linkage between both ETH10 and CSSM34 and  
marbling indicates that one or more loci affecting marbling  
occurs on bovine chromosome 5. The replicated, strong  
25 population association with CSSM34, and the weak  
association to the nearby gene LALBA indicate that a locus  
affecting marbling is closely associated with CSSM34. The  
gene for RARG is closely associated with CSSM34, and  
occurs in the same DNA clone with it. On the basis of  
30 biochemical evidence, RARG is a strong candidate gene for  
the effect, as it is a ligand for all-trans retinoic acid  
(Mertz *et al*, 1997), and the concentration of retinol in  
the serum is directly related to the marbling score of a  
steer (Torii *et al*, 1996), but unrelated to subcutaneous  
35 fat thickness. This indicates that RARG is the likely  
locus affecting marbling. Nevertheless, CSSM34 is not the  
only predictor of marbling score in this genomic region,

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and markers on either side of CSSM34 will also act to predict marbling, just as LALBA is a weak predictor of marbling capacity due to its close proximity. The gene encoding the Roan factor (Charlier et al, 1996) is in the same genomic region as CSSM34, and so, in breeds that segregate the Roan factor, the colour of a steer will be associated with marbling score in some families. These polymorphisms must be assumed to be predicting a locus affecting marbling in and around the RARG gene in cattle.

10           The fact that the Wagyu data (Example 6) show a peak at ETH10, some 20 cM or 54 cR from CSSM34 (Barendse et al, 1997), may indicate that there is more than one gene for marbling on chromosome 5. The ETH10 polymorphism shows no association to marbling in the Angus and Shorthorn, while the CSSM34 polymorphism shows no association to marbling in the Wagyu offspring. The gene RDH5, catalysing the conversion of 11-cis and 9-cis retinol to 11-cis and 9-cis retinoic acid, is extremely closely associated with ETH10. Indeed, the association of ETH10 with RDH5 is closer than that of CSSM34 with RARG. Again, the level of retinol in the serum is directly related to marbling score in cattle, and an enzyme catalyzing the conversion of retinol to retinoic acid would affect the availability of retinoic acid for binding to the retinoic acid receptors. RDH5 is thus a strong candidate for a locus affecting marbling in Wagyu-derived cattle. Other polymorphisms near ETH10 and RDH5 would also show linkage to marbling, and those polymorphisms must be assumed to be predicting the same locus affecting marbling score in and around the RDH5 gene in cattle. The total evidence for a marbling gene on chromosome 5 is convincing, with a combined probability of less than 0.00015 of being due to chance.

RDH5 and RARG should act in concert, and since they are approximately 20 cM apart, it is likely that some animals will have chromosomes that have a favourable allele for marbling at one locus and an unfavourable allele for marbling at the other locus, cancelling each other out.

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Progress to improve marbling would be slow if those animals were used in breeding schemes using conventional methods. However, with the DNA marker tests specified here, it will be a simple matter to breed cattle that have alleles  
5 favourable for marbling at both genes. Naturally, the breeding would also use the TG marker on chromosome 14, which is expected to be associated with fatness in general in addition to marbling, to generate steers of consistent and optimal marbling score.

10

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding,  
15 various modifications and alterations to the embodiments and methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

References cited herein are listed on the  
20 following pages, and are incorporated herein by this reference.



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 10 Livestock Corporation, Sydney) 14-15

# SEQUENCE LISTING

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10 (D) STATE: ACT

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(F) POSTAL CODE (ZIP): 2060

20

(ii) TITLE OF INVENTION: ASSESSING LIPID METABOLISM

(iii) NUMBER OF SEQUENCES: 18

25 (iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

30 (EPO)

(v) CURRENT APPLICATION DATA:

APPLICATION NUMBER: AU PP0120

35

- 45 -

## (2) INFORMATION FOR SEQ ID NO: 1:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 23 base pairs  
5      (B) TYPE: nucleic acid  
        (C) STRANDEDNESS: single  
        (D) TOPOLOGY: linear  
    (ii) MOLECULE TYPE: DNA (genomic)  
    (iii) HYPOTHETICAL: NO  
10     (iv) ANTI-SENSE: NO  
       (vi) ORIGINAL SOURCE:  
            (A) ORGANISM: Bos taurus  
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

15 GGGGATGACT ACGAGTATGA CTG

23

## (2) INFORMATION FOR SEQ ID NO: 2:

- 20     (i) SEQUENCE CHARACTERISTICS:  
        (A) LENGTH: 23 base pairs  
        (B) TYPE: nucleic acid  
        (C) STRANDEDNESS: single  
        (D) TOPOLOGY: linear  
25     (ii) MOLECULE TYPE: DNA (genomic)  
       (iii) HYPOTHETICAL: NO  
       (iv) ANTI-SENSE: NO  
       (vi) ORIGINAL SOURCE:  
            (A) ORGANISM: Bos taurus  
30     (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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23

35

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## (2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 545 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: DNA (genomic)  
(iii) HYPOTHETICAL: NO  
(iv) ANTI-SENSE: NO  
(vi) ORIGINAL SOURCE:  
    (A) ORGANISM: Bos taurus  
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15 GGGGATGACT ACGAGTATGA CTGTGCGTGT GTTTGGCTTA TCTCATCAAA  
ATCTCTACAT 60

TCTGTGTTAA TGGATCTGCC TGTTTTGTTC CCTGCCATAT CCTCATGGCC  
TAGAATAGTG 120

20 TCTGCTTCTC TATCAGACTC TAAAGAAACA TTGCTAGGAG GGAAGGAAGG  
AGCATGGATG 180

AGGAGGGAGG GAGCATTGTG TTTCTCTCAC GGTGGGCCTG AACGTGTGGC  
25 CCACCAAGTT 240

GTTAACTTTG GCCTTTACCC CTGAAGATGA ATTATGAAGC CACACCCCCA  
GTTCTTCCTT 300

30 GGTGGCTCAG ATGGTCAAGA ATCCACCTGC AATGCGGGAG ACCTGGGTTT  
GATCCCTGGG 360

TTGGGAAGAT CCCCTGGAGA AGGGAATGGC TACCCACTCC AGTATTCTGG  
CCTGGAGAAT 420

35 CCCATGGACA GAGGAGCCTG GCGGGATGCA GTCCATGGGG TCTCAGAGAG  
TCAGATGTGA 480



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CTGAGCGACT TTCACACACA CTCGTCCCTG GTTCTGCTCC CCTACAGCCT  
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5 TTCAC 545

(2) INFORMATION FOR SEQ ID NO: 4:

- 10 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 545 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- 15 (ii) MOLECULE TYPE: DNA (genomic)  
(iii) HYPOTHETICAL: NO  
(iv) ANTI-SENSE: NO  
(vi) ORIGINAL SOURCE:  
(A) ORGANISM: Bos taurus
- 20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GGGGATGACT ACGAGTATGA CTGTGCGTGT GTTTGGCTTA TCTCATCAAA  
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25 TCTGTGTTAA TGGATCTGCC TGTTTTGTTC CCTGCCATAT CCTCATGGCC  
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TCTGCTTCTC TATCAGACTC TAAAGAAACA TTGCTAGGAG GGAAGGAAGG  
AGCATGGATG 180

30 AGGAGGGAGG GAGCATTGTG TTTCTCTCAC GGTGGGCCTG AACGTGTGGC  
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GTTAACCTTTG GCCTTTACCC CTGAAGATGA ATTATGAAGC CACACCCCCA  
35 GTTCTTCCTT 300

- 48 -

GGTGGCTCAG ATGGTCAAGA ATCCACCTGC AATGCGGGAG ACCTGGGTTT  
GATCCCTGGG 360

TTGGGAAGAT CCCCTGGAGA AGGGAATGGC TACCCACTCC AGTATTCTGG  
5 CCTGGAGAAT 420

CCCATGGACA GAGGAGCCTG GCGGGATGCA GTCCATGGGG TCTCAGAGAG  
TCAGATGTGA 480

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TTCAC 545

15

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

20 (A) LENGTH: 545 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

25 (iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Bos taurus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

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TCTGTGTTAA TGGATCTGCC TGTTTTGTTC CCTGCCATAT CCTCATGGCC  
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35

TCTGCTTCTC TATCAGACTC TAAAGAAACA TTGCTAGGAG GGAAGGAAGG  
AGCATGGATG 180

- 49 -

AGGAGGGAGG GAGCATTGTG TTTCTCTCAC GGTGGGCCTG AACGTGTGGC  
CCACCAAGTT 240

5 GTTAACTTTG GCCTTTACCC CTGAAGATGA ATTATGAAGC CACACCCCCA  
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GGTGGCTCAG ATGGTCAAGA ATCCACCTGC AATGCGGGAG ACCTGGGTTT  
GATCCCTGGG 360

10 TTGGGAAGAT TCCCTGGAGA AGGGAATGGC TACCCACTCC AGTATTCTGG  
CCTGGAGAAT 420

CCCATGGACA GAGGAGCCTG GCGGGATGCA GTCCATGGGG TCTCAGAGAG  
15 TCAGATGTGA 480

CTGAGCGACT TTCACACACA TTCGTCCCTG GTTCTGCTCC CCTACAGCCT  
CCACAAGATT 540

20 TTCAC 545

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- 25 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 24 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- 30 (ii) MOLECULE TYPE: DNA (genomic)  
(iii) HYPOTHETICAL: NO  
(iv) ANTI-SENSE: NO  
(vi) ORIGINAL SOURCE:  
(A) ORGANISM: Bos taurus
- 35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CCATAACTCT GGGACTTTTC CTCA 24

- 50 -

## (2) INFORMATION FOR SEQ ID NO: 7:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 24 base pairs  
5      (B) TYPE: nucleic acid  
        (C) STRANDEDNESS: single  
        (D) TOPOLOGY: linear  
    (ii) MOLECULE TYPE: DNA (genomic)  
    (iii) HYPOTHETICAL: NO  
10     (iv) ANTI-SENSE: NO  
       (vi) ORIGINAL SOURCE:  
            (A) ORGANISM: Bos taurus  
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

15   ATGTTTCAGCC ATCTCTCCTT GTCC                   24

## (2) INFORMATION FOR SEQ ID NO: 8:

- 20     (i) SEQUENCE CHARACTERISTICS:  
        (A) LENGTH: 931 base pairs  
        (B) TYPE: nucleic acid  
        (C) STRANDEDNESS: single  
        (D) TOPOLOGY: linear  
25     (ii) MOLECULE TYPE: DNA (genomic)  
       (iii) HYPOTHETICAL: NO  
       (iv) ANTI-SENSE: NO  
       (vi) ORIGINAL SOURCE:  
            (A) ORGANISM: Bos taurus  
30     (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

TATGATACAA TTCGAGCTCG GTACCTACAT GTTCCCAAGG ATGCTAATGA  
AGATCACTGA       60

35   CCTCCGGGGC ATCAGCACCA AGGGTTAGTC GGGAGCAAGC CTCCCCTCTG  
TCTTCTCGGA       120

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GCTGCCGGTC TCCCAGGTCA GGCAGAGACA AGAGCAAGTG GGGTATAATC  
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15

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20 GCAGAGAAGA 540

CAGGCAGAGT TCCAGGCAGA GGAGTGGGCC CCAGGAGCTT TACAGTAGAA  
AGAGGGAGAG 600

25 AAAGAAGCAG ACAGAGATAA CAGGCCTGTG ATGGGAGCCC CAGAGGGCAG  
TCAAGCAGAG 660

TTAGGGAGGC CGCCGTAGGT GCTGTACTCA GCCCCCTGAA CTCTTGTTCT  
CCTGTCAGG 720  
30

AGCAGAAAGG GCCATTACCC TGAAGATGGA GATTCCAGGC CCGATGCCTC  
CCCTGATCCG 780

AGAAATGCTG GAGAACCCCG AAATGTTTGA GGACGACTCC TCGCAGCCTG  
35 GCCCTCACCC 840

- 52 -

CAAGGCCTCT AGCGAGGATG AGGTTCTCTG GGATCCTCTA GAGTCGACCT  
GCAGGCATGC 900

AAGCTAGGCA CTGGCCGTCG TTTTACAACA A 931

5

(2) INFORMATION FOR SEQ ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:
- 10 (A) LENGTH: 23 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- 15 (iii) HYPOTHETICAL: NO  
(iv) ANTI-SENSE: NO  
(vi) ORIGINAL SOURCE:  
(A) ORGANISM: Bos taurus
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:
- 20 CCAAGGATGC TAATGAAGAT CAC 23

(2) INFORMATION FOR SEQ ID NO: 10:

25

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 23 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single
- 30 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO  
(iv) ANTI-SENSE: NO  
(vi) ORIGINAL SOURCE:
- 35 (A) ORGANISM: Bos taurus

- 53 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GACTAACATT CATCAAACAC CGC 23

5

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

10 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

15 (iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Bos taurus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

20 CCGCGACAAA AACTGTATCA 20

(2) INFORMATION FOR SEQ ID NO: 12:

25 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Bos taurus

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

TTGCTGACCT TGGTGATGAG 20

- 54 -

## (2) INFORMATION FOR SEQ ID NO: 13:

- 5       (i)   SEQUENCE CHARACTERISTICS:  
          (A) LENGTH: 20 base pairs  
          (B) TYPE: nucleic acid  
          (C) STRANDEDNESS: single  
          (D) TOPOLOGY: linear
- 10       (ii)  MOLECULE TYPE: DNA (genomic)  
          (iii) HYPOTHETICAL: NO  
          (iv)  ANTI-SENSE: NO  
          (vi)  ORIGINAL SOURCE:  
              (A) ORGANISM: Bos taurus
- 15       (xi)  SEQUENCE DESCRIPTION: SEQ ID NO: 13:

AATCCGAGAG ATGCTGGAGA                   20

## 20   (2) INFORMATION FOR SEQ ID NO: 14:

- (i)   SEQUENCE CHARACTERISTICS:  
              (A) LENGTH: 20 base pairs  
              (B) TYPE: nucleic acid
- 25       (C) STRANDEDNESS: single  
          (D) TOPOLOGY: linear
- (ii)  MOLECULE TYPE: DNA (genomic)  
          (iii) HYPOTHETICAL: NO  
          (iv)  ANTI-SENSE: NO
- 30       (vi)  ORIGINAL SOURCE:  
              (A) ORGANISM: Bos taurus
- (xi)  SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CACCCCTAGA AACTTTGGCA                   20

35



- 55 -

(2) INFORMATION FOR SEQ ID NO: 15:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 19 base pairs  
5 (B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: DNA (genomic)  
(iii) HYPOTHETICAL: NO  
10 (iv) ANTI-SENSE: NO  
(vi) ORIGINAL SOURCE:  
(A) ORGANISM: Bos taurus  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

15 ATGCCAAGCT GCTCTGGTT 19

(2) INFORMATION FOR SEQ ID NO: 16:

- 20 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 24 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- 25 (ii) MOLECULE TYPE: DNA (genomic)  
(iii) HYPOTHETICAL: NO  
(iv) ANTI-SENSE: NO  
(vi) ORIGINAL SOURCE:  
(A) ORGANISM: Bos taurus
- 30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

TGAAGTGACT GTTTTATGCC ACAC 24

35

- 56 -

## (2) INFORMATION FOR SEQ ID NO: 17:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 22 base pairs  
5      (B) TYPE: nucleic acid  
        (C) STRANDEDNESS: single  
        (D) TOPOLOGY: linear  
    (ii) MOLECULE TYPE: DNA (genomic)  
    (iii) HYPOTHETICAL: NO  
10     (iv) ANTI-SENSE: NO  
       (vi) ORIGINAL SOURCE:  
           (A) ORGANISM: Bos taurus  
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

15   TTCAGGACTG GCCCTGCTAA CA                   22

## (2) INFORMATION FOR SEQ ID NO: 18:

- 20     (i) SEQUENCE CHARACTERISTICS:  
        (A) LENGTH: 23 base pairs  
        (B) TYPE: nucleic acid  
        (C) STRANDEDNESS: single  
        (D) TOPOLOGY: linear  
25     (ii) MOLECULE TYPE: DNA (genomic)  
       (iii) HYPOTHETICAL: NO  
       (iv) ANTI-SENSE: NO  
       (vi) ORIGINAL SOURCE:  
           (A) ORGANISM: Bos taurus  
30     (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

CCTCCAGCCC ACTTTCTCTT CTC                   23

CLAIMS

1. A method of assessing the fat metabolism characteristics of an animal, comprising the step of testing the animal for the presence or absence of one or  
5 more markers selected from the group consisting of:
  - a) an allele of the 5' untranslated region of the gene encoding thyroglobulin;
  - b) an allele of the DNA polymorphism CSSM34, associated with the gene encoding retinoic acid receptor  
10 gamma (RARG)
  - c) an allele of the DNA polymorphism ETH10, associated with 11-cis, 9-cis retinol dehydrogenase (RDH5).
2. A method according to Claim 1, comprising the step of testing the animal for the presence or absence of  
15 an allele of the 5' untranslated region of the gene encoding thyroglobulin.
3. A method according to Claim 2, in which the allele is allele 3, which indicates a high marbling score and/or high fat content of milk.
- 20 4. A method according to Claim 2, in which the allele is allele 2, which indicates a low marbling score and/or low fat content in milk.
5. A method according to Claim 1 of identifying an animal with a high propensity for fat deposition in muscle  
25 (high marbling score), comprising the step of testing said animal for the presence or absence of allele 3 of the 5' untranslated region of the gene encoding thyroglobulin, and selecting those animals possessing the allele.
6. A method according to Claim 5, in which the  
30 animal is also tested for the presence or absence of allele 2 of the 5' untranslated region of the gene encoding thyroglobulin, and those animals possessing allele 3 and not possessing allele 2 are selected.
7. A method according to Claim 5 or Claim 6, in  
35 which the animal is homozygous for allele 3.
8. A method according to Claim 1 of identifying an animal with a low propensity for fat deposition in muscle,

comprising the step of testing the animal for the presence or absence of allele 2 of the 5' untranslated region of the gene encoding thyroglobulin, and selecting those animals having allele 2.

- 5 9. A method according to Claim 8, in which the animal is also tested for allele 3, and those animals having allele 2 but not allele 3 are selected.
- 10 10. A method according to Claim 8 or Claim 9, in which the animal is homozygous for allele 2.
- 11 11. A method according to Claim 1 of identifying an animal with a high propensity for fat deposition in muscle (high marbling score), comprising the step of testing the animal for the presence or absence of an allele of the DNA polymorphism CSSM34 associated with the gene encoding  
15 retinoic acid receptor gamma (RARG).
- 12 12. A method according to Claim 11, in which the allele is allele 2.
- 13 13. A method according to Claim 12, in which the animal is also tested for other alleles at the CSSM34 DNA  
20 polymorphism.
- 14 14. A method according to Claim 12 or Claim 13, in which the animal is homozygous for allele 2.
- 15 15. A method according to Claim 1 of identifying an animal with a low propensity for fat deposition in muscle  
25 (low marbling score), comprising the step of testing the animal for the presence or absence of an allele of the DNA polymorphism CSSM34 associated with the gene encoding retinoic acid receptor gamma.
- 16 16. A method according to Claim 15, in which the  
30 animal is also tested for other alleles at the CSSM34 DNA polymorphism.
- 17 17. A method according to Claim 16, in which the animal is homozygous for allele 6.
- 18 18. A method according to Claim 1 of identifying an  
35 animal with intermediate propensity for fat deposition in muscle (low marbling score), comprising the step of testing the animal for the presence or absence of an allele of the

DNA polymorphism CSSM34 associated with the gene retinoic acid receptor gamma.

19. A method according to Claim 18, in which the allele is one or more of alleles 1, 3, 4, and 5.
- 5 20. A method according to Claim 19, in which the animal is also tested for other alleles at the CSSM34 DNA polymorphism.
21. A method according to Claim 1 of identifying an animal of, or derived from, the Wagyu cattle breed with a  
10 high propensity for fat deposition in muscle, comprising the step of testing the animal for the presence or absence of an allele of the ETH10 DNA marker.
22. A method according to Claim 21, in which the allele is allele 5.
- 15 23. A method according to Claim 1 of identifying an animal of, or derived from, the Wagyu cattle breed with a low propensity for fat deposition in muscle, comprising the step of testing the animal for the presence or absence of an allele of the ETH10 DNA marker.
- 20 24. A method according to Claim 23, in which the allele is allele 2.
25. A method according to any one of Claims 1 to 24, in which animals are selected for high or low fat content of milk or for high or low fat levels in carcasses.
- 25 26. A method of detecting one or more alleles selected from the group consisting of the 5' untranslated region of thyroglobulin or of retinoic acid receptor gamma, and in an animal, comprising the steps of:
  - a) obtaining a biological sample from the  
30 animal,
  - b) extracting DNA from the sample,
  - c) amplifying DNA from the relevant gene, and
  - d) identifying alleles in the amplified DNA.
27. A method according to Claim 26, in which the  
35 biological sample is blood.
28. A method according to Claim 27, in which the allele is of the 5' untranslated region of the

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thyroglobulin gene, and the region of DNA amplified includes a homopurine sequence and a copy of the monomeric dispersed repeat sequence.

29. An oligonucleotide probe for amplification of the markers of the invention, selected from the group consisting of:

a) oligonucleotide probes for the 5' untranslated region of the thyroglobulin gene, having the sequences

10

TG5U2 5' ggg gat gac tac gag tat gac tg 3' (SEQ ID NO: 1)

TG5D1 5' gtg aaa atc ttg tgg agg ctg ta 3' (SEQ ID NO: 2)

15

b) oligonucleotide probes for amplification of the CSSM34 DNA marker, with the sequences

CSSM34U 5' cca taa ctc tgg gac ttt tcc tca 3' (SEQ ID NO. 6)

CSSM34D 5' atg ttc agc cat ctc tcc ttg tcc 3' (SEQ ID NO. 7)

20

c) oligonucleotide probes for amplification of fragments from the RARG gene in cattle, with sequences

RARGSJ1U 5' cca agg atg cta atg aag atc ac 3' (SEQ ID NO: 9)

RARGSJ1D 5' gac taa cat tca tca aac acc gc 3' (SEQ ID NO 10)

25

RARGE3U1 5' ccg cga caa aaa ctg tat ca 3' (SEQ ID NO: 11)

RARGE3D1 5' ttg ctg acc ttg gtg atg ag 3' (SEQ ID NO: 12)

RARGE8U2 5' aat ccg aga gat gct gga ga 3' (SEQ ID NO: 13)

RARGE8D1 5' cac ccc tag aaa ctt tgg ca 3' (SEQ ID NO: 14)

30

d) oligonucleotide probes for amplification of fragments from the RDH5 gene in cattle, with sequences

RDH5U 5' atg cca agc tgc tct ggt t 3' (SEQ ID NO: 15)

RDH5D 5' tga agt gac tgt ttt atg cca cac 3' (SEQ ID NO: 16)

35

e) oligonucleotide probes for amplification of the ETH10 marker in Wagyu cattle, with sequences:

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ETH10U 5' gtt cag gac tgg ccc tgc taa ca 3' (SEQ ID NO: 17)

ETH10D 5' cc tcc agc cca ctt tct ctt ctc 3' (SEQ ID NO: 18)

- 5 30. A method according to any one of Claims 26 to 28, in which the amplification is performed using a probe according to Claim 29.
31. A Yeast Artificial Chromosome library, grid reference points 77D3, 77E3, 71G8, 94B4 and 71E4, which is
- 10 positive by hybridization to the oligonucleotide primers for CSSM34U and CSSM34D as well as to those for RARGE8U2 and RARGE8D1.
32. An isolated nucleic acid molecule encoding bovine retinoic acid receptor gamma, comprising the sequence set
- 15 out in SEQ ID NO: 8, or hybridizing thereto under stringent conditions.

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33 22 23 23 22 22 23 22 22 23 13

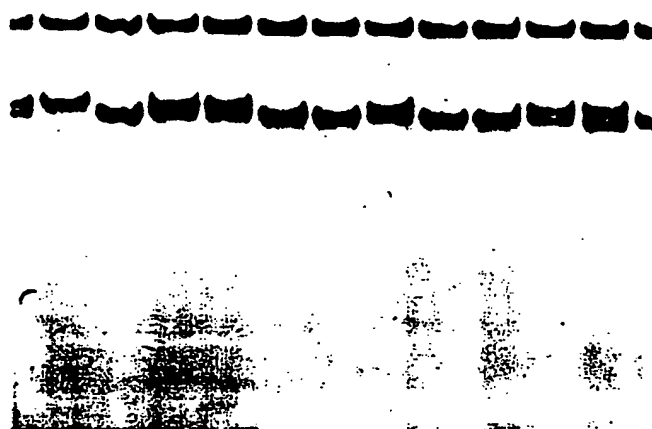
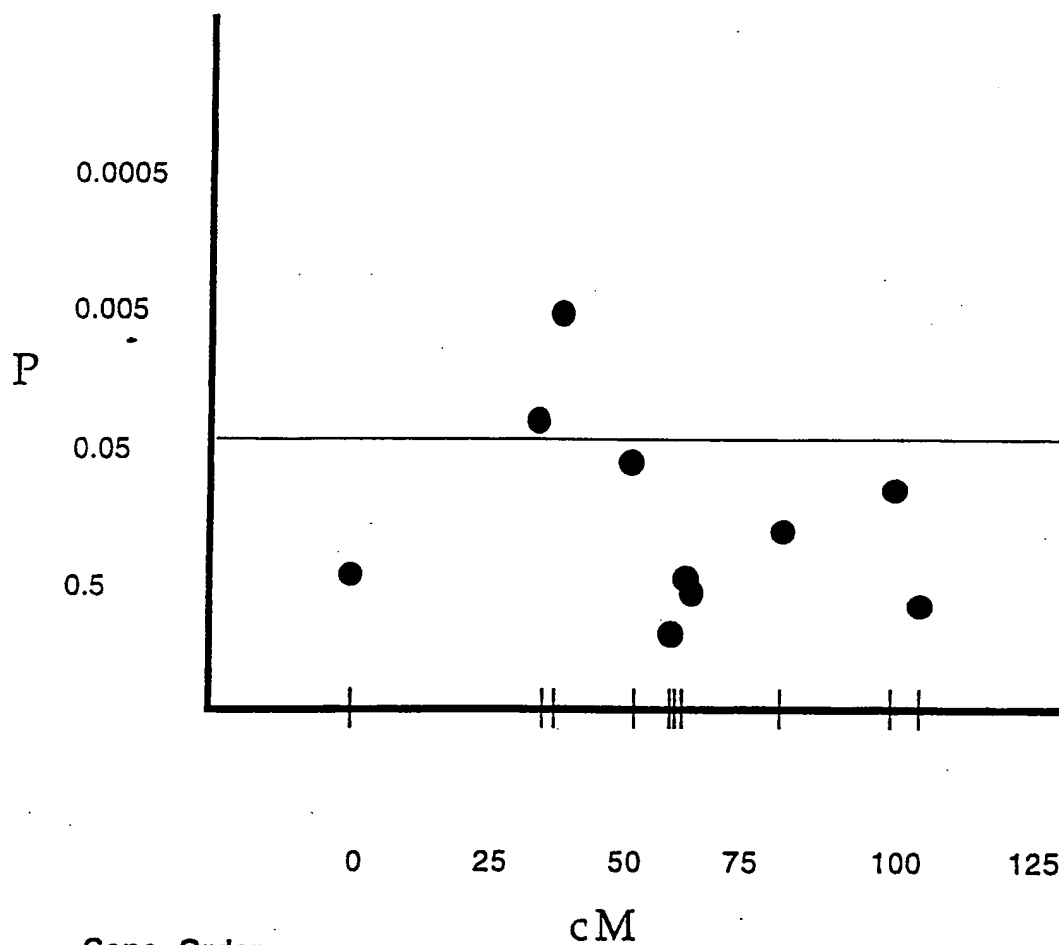


FIGURE 1



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Tests of Association between DNA markers  
on chromosome 5 and marbling



Gene Order

BM6026\*  
LALBA  
CSSM34  
RM500  
ETH10  
RM29  
CSSM22  
AGLA254  
BM315  
ETH2



Strong association at location of RARG

Location of RDH5

\*: Yates correction for continuity due to many alleles with small expectations

FIGURE 2

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/AU 98/00882

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>		
Int Cl <sup>6</sup> : C12Q 1/68, C12N 15/12		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b>		
Minimum documentation searched (classification system followed by classification symbols)		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) DERWENT WPAT, MEDLINE, BIOSIS KEYWORDS: THYROGLOBULIN, RETINOIC ACID RECEPTOR, RETINOL DEHYDROGENASE, FAT, MILK		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 90/15815 A (SALK INSTITUTE) 27 December 1990 Whole document	32
Y	US 5612179 A (SIMONS) 18 March 1997 Whole document	26-28
Y	MAMMALIAN GENOME, vol 8, 1997, pages 21-28, BARENDSE W, ET AL, "A medium density genetic linkage map of the bovine genome" page 24, Table 2	26-28
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C <input checked="" type="checkbox"/> See patent family annex		
<p>* Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier application or patent but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p>		
Date of the actual completion of the international search 13 November 1998		Date of mailing of the international search report <b>19 NOV 1998</b>
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929		Authorized officer  <b>ROSS OSBORNE</b> Telephone No.: (02) 6283 2404

**INTERNATIONAL SEARCH REPORT**

International application No.

**PCT/AU 98/00882**

<b>C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
<b>Category*</b>	<b>Citation of document, with indication, where appropriate, of the relevant passages</b>	<b>Relevant to claim No.</b>
<b>Y</b>	EUR. J. BIOCHEM vol. 165, No 3, (1987) p 491-498, MALTHIERY Y. ET AL, "Primary structure of thyroglobulin deduced from the sequence of its 8448 base complimentary DNA." Whole document	26-28
<b>A</b>	US 5614364 A (TUGGLE ET AL) 25 March 1997 Whole document	1-32
<b>A</b>	WO 92/13102 A (GENMARK) 6 August 1992 Whole document	1-32

## INTERNATIONAL SEARCH REPORT

### Information on patent family members

International application No.  
**PCT/AU 98/00882**

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report				Patent Family Member			
WO	9213102	AU	13752/92	CA	2100583	EP	570496
WO	9015815	AU	60519/90	CA	2066692	EP	479916
		US	5260432	US	5530094		
US	5612179	AU	61319/90	AU	72850/94	CA	2023888
		DD	299319	EP	414469	IL	95467
		JP	3139300	NZ	235051	SG	47747
		ZA	9006765	US	5192659	US	5789568
US	5614364	NONE					
END OF ANNEX							